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BIOCHEMICAL AND GENETIC MECHANISMS IN REGULATION PROCESSES IN THE BACTERIAL CELL

[Following is a translation of an article by Francois Jacob and Jacques Monod, of the Departments of Microbial Genetics and Cellular Biochemistry of the Pasteur Institute, Paris, in the French-language journal Bulletin de la Societe de Chimie Biologigne (Bulletin of the Society of Biological Chemistry), Vol XLVI, No 12, Paris, 1964, pages 1499-1532.]

946

I. Introduction

The knowledge which has been acquired over the past years on the structure of the essential biological macromolecules, i.e., nucleic acids and proteins, has made us understand at least in large general lines, the relationship of the functions of these macromolecules and their chemical structure. The study of DNA replication in vivo and in vitro has shown that the fundamental chemical mechanism of heredity is very well the one which has been proposed by Watson and Crick (1953) after their discovery of the structure of DNA. The discovery of messenger RNA and its role in the biosynthesis of proteins, the study of the coding process, and research on the genetic determinism of the primary structures of the proteins, have wholly confirmed and reconfirmed the older hypotheses on the relationships between genetic determinants and protein-enzymes.

The progress which has been made in biochemistry during the last 50 years has shown, among other things, that the properties, structures, and activities which characterize a cell should definitely be attributed to the structure and activity of the proteins which this cell is capable of synthesizing. The structure of each one of these proteins is well defined, and it can today be confirmed by a segment

containing hereditary characteristics. But since the structure of the DNA of a cell or a cell strain does not change, the biochemical properties which are expressed by this cell could well be profoundly different; besides, these properties can be changed as a function of the specific chemical signals which come from the outside. The injection of thyroid hormone into a tadpole can cause, before any other morphological changes appear, a conversion of its excretory system which, resembling the one in fishes, can become chemically analogous to that of the mammal. Several hours after the injection, the specific enzymes of the urea cycle increase in the proportion of fifty to a hundred times and the animal will excrete urea, even though up to this point it had excreted only ammonia. Escherichia coli, when cultivated in a medium containing maltose and histidine, synthesized amylose but not histidine. The same clone growing in a medium containing lactose and tryptophane will not synthesize amylose, but it will synthesize histidine. It will not, however, synthesize tryptophane, and enzymatic studies would show that in the first medium this bacterium would synthesize amylomaltase and phosphorylase, but only traces of nine enzymes which, coming from ATP and ribosylpyrophosphate, would participate in the synthesis of histidine. When they were placed in the second medium, these same bacteria did not synthesize amylomaltase nor phosphorylase any longer but, on the other hand, produced galactosidase and the series of enzymes which are involved in the synthesis of histidine.

One could then doubt, and it has in fact been doubted for a long time, that the chemical activities which are also directly attributed to the conditions of the medium could, at the same time, be specifically and precisely governed by these invariants, the genes, or, to state it in another way, by the structure of DNA.

This apparent paradox is partially resolved today. Studies of the control mechanisms of cellular metabolisms have revealed the existence of a complex of specific molecular interactions which have the function of regulating and coordinating the activity and biosynthesis of the cellular proteins. In this present article we would like to demonstrate and emphasize the research and knowledge which has been accumulated on this subject. In trying to treat this problem as a whole, it will not be possible for us to go into much detail.

We shall divide this article into three sections which will correspond to the three levels of increasing complexity in cellular regulation:

1. Regulation of enzyme activity
2. Regulation of protein biosynthesis
3. Regulation of cell division

II. Primary Regulatory Interaction: Allosteric Effects

In 1938, Cori and his school (Cori et al., 1938; Cori and Green, 1943) isolated phosphorylase from rabbit muscle and showed that this enzyme was practically inactive in the absence of a specific effector, namely 5' AMP. AMP had naturally been considered up to this time as playing the role of a "coenzyme" in the reaction of phosphorylase b. But later research has shown that this nucleotide actually does not participate in the reaction which is really catalyzed by phosphorylase, and does not therefore play a role as a coenzyme in the sense in which we understand it today (see Krebs and Fischer, 1962). Over these last years, an increasing number of enzymatic systems have been shown to be sensitive to the activating or inhibiting effects of specific metabolites; in the majority of cases, these effects could be clearly interpreted from physiological and functional points of view. It is easy to see that the activation of acetyl-CoA-carboxylase by citric acid (Martin and Vagelos, 1962), the inhibition of citrate synthesis by glucose-6-phosphate (Traut and Lipmann, 1963), can and should play an important role in the regulation of energetics metabolism.

Even clearer from a physiological standpoint are the properties of certain bacterial enzymes which participate in the synthesis of the essential metabolites (see Umbarger, 1961). It is known, for example, that aspartyl-transcarbamylase intervenes as a primary enzyme in the sequence of reactions leading to the synthesis of pyrimidine nucleotides. This enzyme, which is specifically inhibited by CTP', is also specifically activated by ATP (Gerhart and Pardee, 1962). It is certain that these remarkable properties, which are highly specific, do not play an essential role in the regulation of pyrimidine synthesis and in the coordination of the synthesis of the purine and pyrimidine nucleotides. It is also known that threonine-deaminase intervenes in the primary reaction leading to the synthesis of isoleucine. The activity of this enzyme, which is inhibited by isoleucine (Umbarger, 1956), is activated by valine (Changeux, 1961): this situation is entirely analogous to that of ATCase and can be interpreted physiologically the same way.

These remarkable properties which, at the time of their discovery, appeared quite exceptional, are in fact examples of a general rule which one could state in the following manner: in most, if not all metabolic pathways, a certain number of enzymes play a specific regulatory role. Their activity is governed not only by the concentration of their substrate, but, at the same time, by other metabolites which can be in the form of products or precursors which may be closely or distantly related to the metabolic pathways in which this enzyme plays a function, or by intermediates or products of a parallel metabolic pathway. It seems that this is due to the particular properties of these regulatory enzymes which are at every instant regulated, as far as absolute or relative amount goes, by the metabolism; as a result of specific interactions which occur on the proteins, a definite homeostasis is maintained in the cell.

But even if the physiological interpretations of these effects is clear, and even if their role appears to be of extreme importance, it would seem, on first sight, to pose a veritable chemical paradox. Comparisons of observations made on a certain number of these regulatory enzymatic systems lead to the conclusion that the specific activating or inhibiting effects in question are a result of indirect interactions between distinct stereospecific receptors which are carried by the same enzymatic protein molecule (Changeux, 1961; Gerhart and Pardee, 1962). The term "allosteric" has been proposed to characterize these effects as well as the proteins in which these are shown (Monod and Jacob, 1961; Monod et al., 1963).

Different observations show, among other things, that the allosteric effects are generally linked to the reversible modifications of the conformations of the proteins, or allosteric transitions. In the majority of the cases, if not in all of them, it seems that the allosteric proteins are oligomeres containing 2, 4 or more identical subunits. In the majority of the cases, experiments suggest or show that the modifications of conformations associated with the allosteric effect are related particularly to the forces which tie these sub-units together. Finally, the kinetics of allosteric effects present certain remarkable characteristics. This whole set of observations, which we cannot discuss here, has led to the proposal of the following model for these allosteric transitions (Changeux et al., 1964).

1. The allosteric proteins are, in general, oligomeres which contain many or several identical sub-units called protomeres containing non-covalent bonds which are distributed in such a fashion that each amino acid residue forming

a bond between protomeres, contributes just as much as the one representing the protomere to which it belongs. As one can easily see, this implies that the proteins possess at least one symmetrical axis.

2. One and only one receptor per protomere corresponds to each bond which is capable of associating specifically with the proteins.

3. Two or more distinct conformational states are reversibly accessible to these proteins. These states are particularly differentiated as follows:

a. The number and distribution of bond energies between the protomeres;

b. Their affinity in regard to one or many stereospecific bonds.

4. When the protein passes from one conformational state to another, its symmetry is still maintained.

According to this model, an allosteric dimer would be able to adopt two, or at least two, distinct conformational states (P and P'), both of which are symmetrical, and an equilibrium would exist between them which could be symbolized as follows:

$$PP \rightleftharpoons P'P'$$

If one of these states (PP) has an affinity for the substrate S, and the other one an affinity for effector F, one would have, among others, the following equilibrium:

a. for the substrate:

$$PP + S \rightleftharpoons PP_s$$
$$PP_s + S \rightleftharpoons {}_sPP_s$$

b. for the effector:

$$P'P' + F \rightleftharpoons P'P'_F$$
$$P'P'_F + F \rightleftharpoons {}_FP'P'_F$$

It is clear that the effector in such a system would behave like a "competitive" inhibitor of the substrate. On

the other hand, a bond which has, like the substrate, a higher affinity for the P conformation than for the P'-conformation would be an activator of the system. In other words the substrate like the effector or effectors tends to associate cooperatively with the protein. A classic example of such a cooperative effect is the interaction heme-heme of hemoglobin (cf. Wyman, 1948, 1963; Muirhead and Perutz, 1963). It now seems that these cooperative effects are the rule for the allosteric systems in general

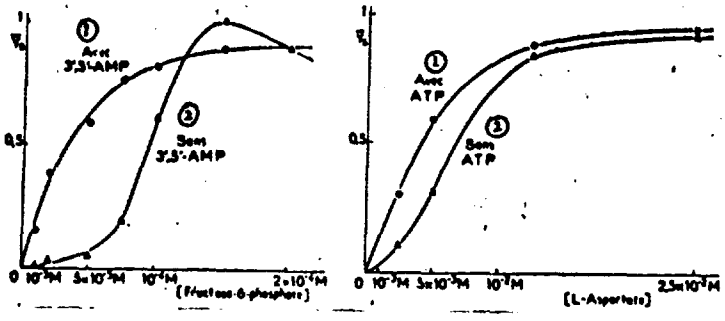

Fig. 1. The action of an allosteric effector on the variation of initial rates of the catalyzed reactions by phosphofructokinase and aspartate transcarbamylase.

[Legend]: 1) with; 2) without.

The initial rates of each reaction are expressed as a fraction ($\overline{Y}_s$) of the maximum rates which are expressed as a function of substrate concentration, fructose-6-phosphate or l-aspartate concentration. The cooperative interactions of the substrate disappear in the presence of the positive effector 3'-5' AMP for phosphofructokinase and ATP for aspartate transcarbamylase. (Adapted from Mansour (1963) for the phosphofructokinase and after Gerhart and Pardee (1962) for the aspartate transcarbamylase).

Figures 1 and 2 give some examples concerning the various allosteric enzymatic systems. Here we are satisfied with these illustrations without trying to develop or justify the proportions serving as the basis for the model. We

note however, that certain of these hypotheses -- in particular those according to which the protein oligomeres have a symmetrical axis -- are probably of quite general application.

We would like particularly to underline here the functional properties of allosteric interactions. Since these interactions are indirect, and there specificity is exclusively governed by the structure of the protein and the conformational states which are accessible to them, these interactions are independent of all necessary relations of structure or of chemical reactivity between the substrates, the allosteric enzymes and the effectors which modify the activity of these enzymes by stabilizing one or another conformational state. It is the absence of a relationship or of chemical reactivity between the substrate and the effector of an allosteric protein, combined with the fact that the association of the effector with the protein is generally not covalent and reversible, which permits these proteins to play their proper regulatory role.

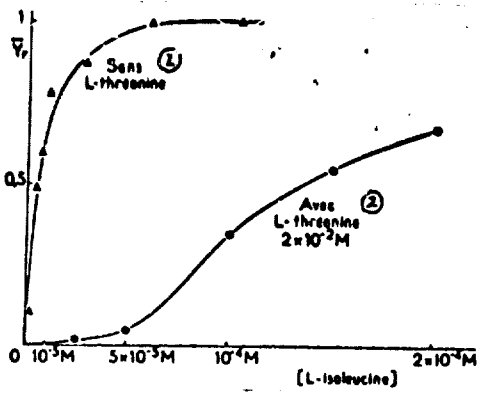

Fig. 2. The action of a substrate on the fraction of receptors saturated by an allosteric effector in the case of threonine-deaminase in E. coli K12.

[Legend]: 1) without; 2) with.

The values of the fraction of the saturated receptors by the l-isoleucine ($\bar{Y}_f$) corresponding to the measured initial rates are expressed as a function of the enzyme. In the absence of the substrate, an estimate of $\bar{Y}_f$ depends on the

protector effect shown by the l-sioleucine on the thermal inactivation of the enzyme. The cooperative interactions of l-sioleucine, which is the negative effector, are not slowed down in the presence of the substrate (cf. Changeux et al., 1964).

It is clear that the regulation of cellular metabolism demands precisely that the activity of such a metabolic pathway be coordinated with other reaction sequences in which the intermediates or the products themselves can actually be chemically foreign to the original ones. As a result of the interactions which the allosteric proteins relay, these proteins permit the establishment of these indispensable connections, and it is clear that all the physiological coordination necessary or simply favorable could be established in the course of biochemical evolution by the selection of the structures accessible to an allosteric protein (see figure 3).

It is in this sense that one can, we believe, consider the allosteric enzymes as constituents of the models of the primary regulatory interactions. In the remainder of this paper we shall see that other regulating effects are expressed not only at the level of control of enzyme activity, but also at the level of the protein synthesis or cell division. Whatever the complexity of these mechanisms may be, they are definitely governed by the metabolites acting not so much by participating themselves in the mechanisms which they govern, but rather by representing specific chemical signals. Consequently, the primary regulatory interaction implies that a specific protein and accompanying modification with properties of the protein recognize the metabolite.

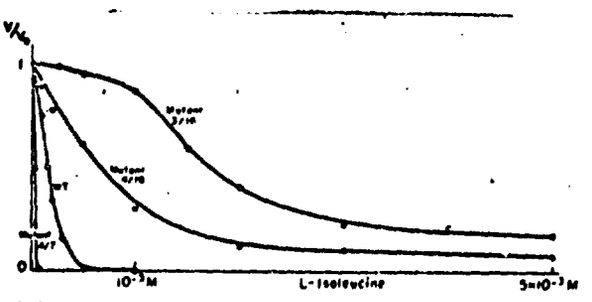

Fig. 3. Inhibition of the activity of threonine-deaminase formed by various mutants of E. coli K12

initial reaction rates expressed as a fraction of the maximum rate are expressed as a function of the concentration of the allosteric inhibitor (l-isoleucine) in the wild type (WT) and in different mutants resulting from the same gene governing the structure of the enzyme (Monod et al., 1963).

Such metabolic interactions are illustrated in the bacteria by mechanisms governing the pathways of biosynthesis of the essential metabolites (see figure 4). Because of a retroactive double turn in the metabolic cycle, the final product of a biosynthetic chain of reactions, for example an amino acid, inhibits the activity of a primary enzyme by the allosteric effect and the synthesis of the sequence of enzymes necessary to its proper biosynthesis. In all probability this inhibition of protein synthesis is likewise due to an allosteric effect produced by the final metabolite on another specific protein, or a repressor, which regulates the activity of the genes governing the structure of these enzymes. This is the problem which we are now going to discuss.

III. Regulation of Protein Biosynthesis

When cultivated in the presence of glucose as its only carbon source, Escherichia coli synthesizes only traces of β-galactosidase. In the presence of a β-galactoside, these same bacteria synthesize considerable quantities of this protein (up to 6 or 7% of their total proteins). This is a classical phenomenon and numerous similar examples are known. It would have appeared "natural" enough to those who first observed it at a time when we still knew so little about matters concerning the chemical structure of enzymes and nothing about the genetic control of these structures. It is only in the light of relatively recent progress in genetics and biochemistry that these phenomena have become apparent as posing a problem, is not, in fact, being a paradox. For such a reason one was able to doubt for a certain time that the increase in activity by which the specific induction of an enzyme is expressed, in reality leads to biosynthesis, in specific protein biosynthesis. The use of tracers has eliminated these doubts, while the identification of a specific locus, which is apparently responsible for the formation of the structure of the galactosidase, and the extension of these results to a number of other systems, permits us to state the problem again but in more precise terms.

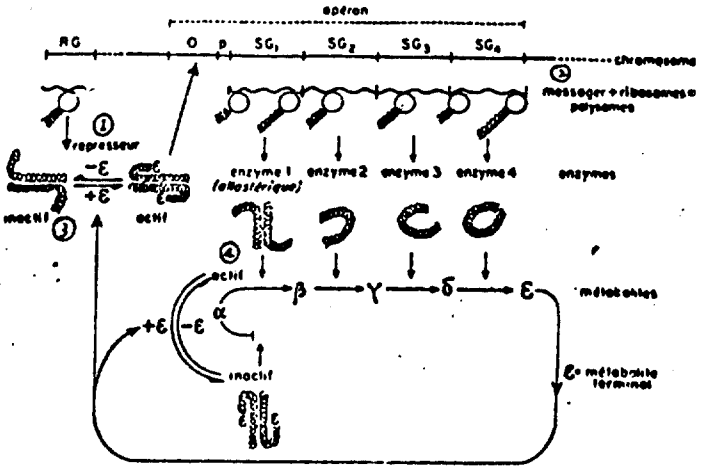

Fig. 4. Schematic representation of regulation governing the biosynthesis of a metabolite in bacteria

[Legend]: 1) repressor; 2) messenger; 3) inactive; 4) active.

A metabolite α is transformed through a series of intermediates β, γ, δ, then into a final product such as an amino acid or base. The reactions are catalyzed by a series of enzymes 1, 2, 3 and 4, the structure of which is determined by the genes SG_1, SG_2, SG_3, and SG_4. These genes are thought to be adjacent on the chromosome and to form a single operon which is transformed into one single messenger, the synthesis of which being governed by an operator o and a promoter p. The production of this messenger is regulated by a repressor is able to combine specifically with the operator and, according to its conformation, can or may not inhibit the production of the messenger by means of the operon. The concentration of the final product of sequence ε gives the system a double regulatory turn.

1) It adjusts the activity of the primary enzyme of the sequence (according to the last metabolic branching leading to the formation of α) by allosteric inhibition.

2) In such a repressible system, the repressor produced by the regulatory gene is thought to be inactive by itself. In the absence of the final product e, the messenger of the operon as well as the enzymes are therefore produced. The metabolite reacts with the repressor and the activator probably by the allosteric effect, which brings about the inhibition of the production of the messenger and, consequently, inhibition of the syntehsis of the enzymes.

In inducible systems, on the other hand, the repressor produced by the regulatory gene is thought to be active by itself. In the absence of the metabolite inducer, neither the messenger of the operon nor the enzymes are therefore produced. The metabolite inducer reacts with the repressor and the inactivator probably by means of an allosteric effect, which brings about the production of the messenger and, consequently, the synthesis of the enzymes.

It was necessary to admit that the expression of a genetic sequence determining the structure of an inducible protein could be governed definitely by the presence of a specific metabolite (cf. Monod, 1956)

While the metabolite in question is a substrate for the enzyme in most cases, it was natural to suppose that the inducing interaction was identical to the association of the substrate with the enzyme itself. But deeper study of the specificity of the induction should likewise eliminate this hypothesis and lead to the conclusion that, in an inducible system, the inductor does not act in such a manner on the substrate (cf. Monod and Cohn, 1952; Monod, 1956, 1959).

Over the same period, a regulatory phenomenon of a type completely different on first sight was discovered. The biosynthesis of the tryptophane-synthetase, an enzyme having a part in the formation of tryptophane (Monod and Cohen-Bazire, 1953; Cohn and Monod, 1953). Very rapidly, this observation was applied to a large number of enzymatic systems (cf. Vogel, 1960). We know today that the biosynthesis of most, if not all, enzymatic systems having a part

in the formation of essential metabolites, is specifically repressed by the metabolite produced by their activity. Again in this instance, experience has shown that the regulatory effect of the metabolite cannot be attributed to a direct interaction with the proteins, the biosynthesis of which it regulates (Gorini and Maas, 1957).

Finally, a third example of the regulatory effect on the synthesis of specific proteins is furnished by the phenomena of immunity in lysogenic bacteria. It is known, in fact, that in these bacteria protein synthesis is determined by the genome of the phage, which is also present in the pro-phage state and is inhibited during the bacterial cell division. Under certain conditions, however, the synthesis of these proteins can be started by the exposure of bacteria to certain agents such as ultraviolet rays or various materials which affect the metabolism of DNA (Lwoff, 1953; Jacob, 1960).

A. General Scheme of Regulation in Protein Synthesis

By detailed genetic and biochemical analysis of mutations which specifically modify these regulatory effects, one could show definitively that the repressible and inducible systems as well as the phenomena of immunity in phage show the same mechanism and thus give these mechanisms a general interpretation (Jacob and Monod, 1961 a; 1963). Our present knowledge on this problem can be illustrated by an overall scheme (see figure 4) and can be summarized as follows:

1. The expression of a gene determining the structure of a protein (structural gene) can be inhibited by a specific macromolecular constituent called a repressor.

2. The repressor itself is a gentic product. A gene determining the structure of a repressor is called a regulatory gene.

3. The repressor can combine specifically with a metabolite. This association is interpreted as an inhibition (inducible system) or as an activation (repressible system) of the repressor.

4. Many structural genes adjacent to each other can be grouped into a unit called an operon. The inhibitory interaction of the repressor in regard to genes belonging to

the same operon, is governed by a particular genetic segment, the operator. The operator is located at one extremity of the segment constituting the operon, but it is distinct from the structural genes.

In the framework of the present article it would be impossible to analyze or even to sum up all of the experimental observations upon which these general conclusions are founded. We shall limit ourselves here to noting briefly the observations which establish the existence of the repressor as a specific genetic product and to adding a little more detail to the basic understanding concerning the operon as a genetic unit governed by an operator. We shall limit this discussion principally to the system of inducible enzymes which insure the utilization of lactose by E. coli.

B. Regulatory Genes and Repressors

The existence of the repressor as a distinct genetic product and both the loci, whose expression it controls, and the proteins determined by these loci, are shown by:

1) the isolation of recessive mutants of the wild type (type i^-, cf. Pardee et al., 1959);

2) the fact that these regulatory loci are distinct from the loci determining the structure of the proteins in question (see figure 5);

3) the isolation, in certain inducible systems, of mutants which are not inducible, resulting from mutations in the same locus as the constituent mutants, that is, mutants characterized by the fact that they are dominant to the wild type (type i^m, cf. Willson et al., 1964).

It is easy to see that such results can be explained only by the hypothesis that the regulatory genes, which are the locus of type i^- and i^S mutations, determine the structure and, therefore, the specificity of the macromolecule, which acts as the repressor and is capable, on the one hand, of inhibiting the expression of an operon, and on the other hand, of recognizing a metabolite in the presence of which the inhibiting property of the repressor is destroyed (in the inducible system). But these conclusions, certain as they may be, pose three problems on a subject about which we still have only indirect knowledge:

a. the chemical nature of the repressor;

b. the nature of the interactions between the repressor and the specific metabolite (inductor or corepressor);

c. the identity of the constituent at the level at which the repressor demonstrates its inhibitory action, that is to say, by definition, the operator.

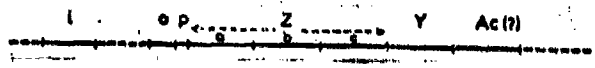

Fig. 5. Representation of the lactose region of E. coli.

i: regulatory gene, o: operator, p: promoter, z: structural gene for the β-galactosidase (represented with 3 cistrons a b c (see text), y: structural gene for galactoside permease, Ac: structural gene for galactoside transacetylase.

The specificity of the induction shows that the repressor-inductor interaction is, to a large extent, stereospecific. The kinetics of the induction show that this interaction is practically instantaneous and suggests that it is reversible (cf. figure 6). Finally, research of the chemical modifications of the inductor, associated with the inductive interaction, has yielded totally negative results up to the present time. These data suggest very strongly that the inductor-repressor interaction consists of a formation of a reversible stereospecific complex, of the type which forms an allosteric protein with an effector. If, among other things, one takes into consideration further the fact that the structure of the repressor is determined by a specific locus, the conclusion seems necessary that the repressor must be, or at least must be composed of, a specific essential element, a protein possessing a specific receptor for the recognition of an effector and in all probability, another receptor for the recognition of the operator. The established properties of known regulatory proteins permit us to theorize that the interaction between these two receptors results in an allosteric transition. It is not possible, of course, that the repressor could possess certain enzymatic properties, but this hypothesis is not absolutely necessary at the present since the inhibitory effect of the repressor could result simply in the capacity to form a complex with the operator. It is with this hypothesis of the

problem that we shall now pause in order to consider the structure of the operon and the nature of the operator.

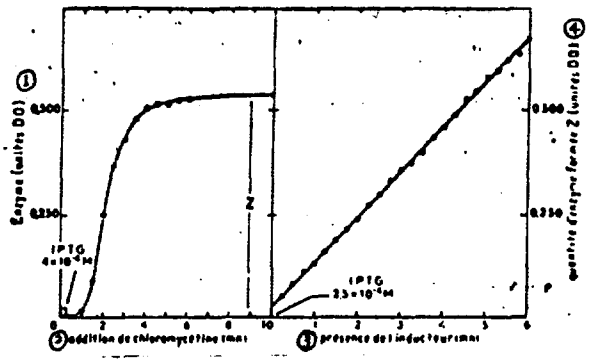

Fig. 6. Kinetics of the induction of β-galactosidase.

[Legend]: 1) units; 2) of; 3) of the inductor; 4) quantity of enzyme Z formed (units DD).

To the left: Production of β-galactosidase following a short induction. 0.4 mM of isopropylthiogalactoside (IPTG) are added at time 0 to a culture of bacteria in the expotential growth phase at a minimum temperature of 37°C. After 20 seconds this culture is diluted 50 times in a preheated medium which does not contain the inductor. Chloramphenicol in a final concentration of 50 μg/ml is added to cultures grown beforehand at various times. The samples are still maintained at 37°C for 20 minutes, then toluene was added and the activity of the β-galactosidase measured. The yield of galactosidase (z) is expressed in the function of time starting at the addition of chloramphenicol.

To the right: The yield of β-galactosidase as a function of the length of the induction. 0.25 mM of IPTG have been added to the bacteria at time 0. After varying times samples are diluted 50 times in a preheated medium containing no inductor. Each sample is maintained at 37°C for 18 minutes, toluene is then added and the activity of β-galactosidase determined. The yield of β-galactosidase

(z) is expressed as a function of the time of contact with the inductor (according to Kepes, 1963).

C. The operon, the messonger coding unit

The existence of the operon as a unit of genetic expression was initially established by the isolation of constituent mutants having the structure of the operator (oc) and by demonstrating that these mutants, contrary to regulatory mutants, affect only the adjacent structural genes in cis position to the operator locus.

Consequently a great number of convergent observations have confirmed the existence of the operon as a unit of expression. Among these we note in particular the fact, which has been demonstrated now in several systems, that the rates of synthesis of the proteins depending on the same operon if the variables are in an absolute value, are invariant in relative value (figure 7). In other words, there exists a quantitative coordination of the expression of genes which belong to the same operon (Ames and Gary, 1959; Jacob and Monod, 1961 b; Burstein et al., 1964). The integral properties which characterize the operon have, on the other hand, been confirmed by the discovery of the so-called "polar mutations" (Jacob and Monod, 1961 b; Franklin and Luria, 1961; Englesberg, 1961; Ames and Hartman, 1963). Let us recall that these result from point mutations, the effects of which are expressed on the one hand by an alteration of the structure of the corresponding protein, and on the other hand, by a decrease of the rate of synthesis of the enzymes, determined by those of the structural genes which belong to the same operon, and are situated farther away than the mutation considered in relation to the operator; the expression of the genes situated between the operator and point mutation is not modified. In the case of the lactose operon for example, the order of the genes is o (operator), z (β-galactosidase comprising several cistrons), y (galactoside-permease) and probably Ac (galactoside-transacetylase) (see figure 5). A polar mutation in y not only results in the inactivation of the permease, but also decreases the rate of synthesis of the transacetylase (distal in relation to the operator) without every time modifying that of the galactosidase (proximal in relation to the operator).

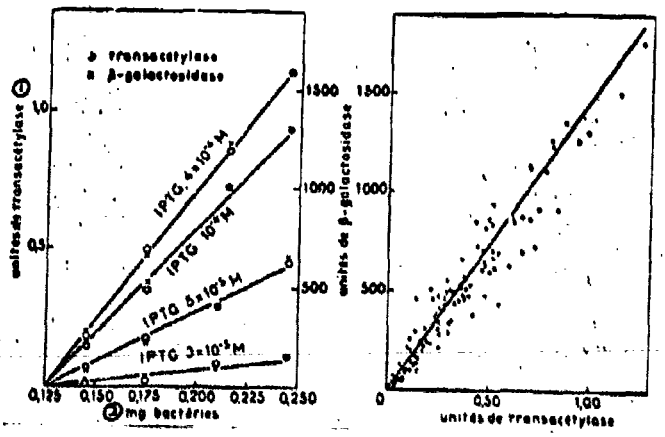

Fig. 7. The coordinated syntehsis of β-galactosidase and of transacetylase under diverse conditions

[Legend]: 1) units of; 2) bacteria.

On the left: A culture of mutants y- (incapable of forming galactoside-permease) is grown on a minimal medium containing glycerol. At time O, isopropyl-thiogalactoside (IPTG) is added in varying concentrations to different fractions of this culture. The bacteria are agitated at 37°C and the β-galactosidase or transacetylase activity is measured on the samples which were removed after varying times. The activities of β-galactosidase or of transacetylase (expressed in units defined by Burstein et al., 1964) are expressed as a function of the weights of the bacteria. One can see that, since the absolute quantities of the enzymes formed vary with the concentration of the inductor, the proportion of the values found does not change.

On the right: the figure sums up the results obtained in numerous experiments with constitutent sources (i^- or o^c), or inducible ones induced under various conditions. Each point represents the β-galactosidase activity as a function of the transacetylase activity (Burstein et al., 1964).

In summary, the existence of the operon as a unit of genetic expression is confirmed by the quantitative coordination of the synthesis of the proteins depending on the same operon and especially through those mutations (o^c and polar) which violate the "one gene-one enzyme" postulate of Beadle and Tatum (1941). It should be emphasized that many of the protein enzymes are composed of many peptide chains. This is the case, for example, with tryptophane synthetase of E. coli (cf. (Yanofsky, 1963). This is likewise true in the case of the β-galactosidase in which the analysis of the partial deletions through complements in vivo and in vitro permits us to demonstrate the existence of many cistrons corresponding to many peptide chains (Villman, Malamy, Jacob and Monod, unpublished results) (see figure 5). If one defines the gene z as the segment of DNA governing the structure of the galactosidase, this gene is polycistronic. It is possible that many operons corresponding to a single gene will appear in reality polycistronic after more detailed analysis.

It remains to be learned, however, how and why the expression of genes belonging to the same operator is integral. It is here that we must mention the theory and the properties which are today recognized concerning messenger RNA.

Let us reiterate again that the same hypothesis according to which the transfer of information between structural gene and the formative center of the proteins (that is to say, the ribosomes) brings in a component which, in bacteria, should more or less have a brief existence and, therefore, should be distinct from ribosomal RNA. This was stated right after the investigations concerning the régulation of β-galactosidase synthesis (Jacob and Monod, 1961 a) and the kinetics of the expression of the corresponding gene, compared to the kinetics of the induction itself (Riley et al., 1960). It is known that this hypothesis, which leads to the postulation of this fraction in vivo (Volkin and Astraghan, 1957; Brenner et al., 1961; Gros et al., 1962). All of these problems concerning the nature and properties of the messenger RNA have been discussed in another connection. We shall only consider here those ideas relating to the interpretation of the integral expression of the operon.

Since the operon constitutes an integral unit of expression, one must ask oneself if the immediate product of the operon should be a messenger, being by itself integral, which is the carrier of information corresponding to the entire sequence of the structural genes composing this operon

(Jacob and Monod, 1961 a). Just a few years ago this hypothesis scarcely appeared probable, considering molecular dimensions, so that it was then necessary to assign the corresponding messenger to certain operons which make up or compose many genes and determine the structure of several enzymes. But recent progress made in extraction and fractionation techniques (Hall and Spiegelman, 1961; Bolton and McCarthy, 1962) has shown that, in fact, the messenger fraction RNA is composed of molecules, the dimensions of which are presumed to be compatible with the existence of messengers capable of integrally transferring information corresponding to 6, 8 or perhaps 10 proteins of average molecular weight (Attardi et al., 1963; Martin, 1963; Spiegelman and Hayashi, 1963; Guttman and Novic, 1963). To this has been added quite recently the demonstration that the expression of the operon can be partially restored by mutations exterior to the operon (Beckwith, 1964 a). It is known that many of these "suppressor" genes of which many examples are known (cf. Yanofsky, 1963; Benzer and Champe, 1961; Campbell, 1961) act, according to all probability, by the intermediates of modifications, which may be activating enzymes, transfer RNA or even of the ribosomal structure itself. In every instance, these mutations do not affect the coding processes (that is to say the biosynthesis of the messenger), but the translating process, that is to say the biosynthesis of the proteins themselves.

These results suggest very strongly that the immediate product of an operon is an integral messenger which associates with many ribosomes in order to form a polysome. The translation of this messenger should follow, and an interruption (or an abatement) of the translation, resulting in a polar point mutation in a structural ciston, should then be followed by an interruption (or the abatement) of the synthesis of enzymes corresponding to the distal cistrons, but not to the proximal cistrons (cf. Beckwith, 1964 a; Ames and Hartman, 1963; Zabin, 1963; Stent, 1964); the proximal extremity of the messenger should, by definition, be the one where peptide syntehsis starts, that is to say according to present knowledge and data (Dintzis, 1961), the N-terminal extremity of the protein determined by the structural gene which is nearest to the operator segment (see figure 10).

But if one can accept as firmly enough established, as we believe it is, that the immediate product of the coding of an operon can be an integral messenger, it remains to be discovered at what level coding or translation is governed by the expression of this operon.

D. Operator, promoter, and the level of action of repressor

All observations show that induction (or depression) of an operon is transformed in particular by accumulation of a specific corresponding messenger. We shall refer here in particular to experiments in which the level of synthesis of specific RNA messengers was raised through hybridization with a corresponding DNA (Attardi et al., 1963; Spiegelman and Hayashi, 1963; Martin, 1963), the results discussed in the report of F. Gros. The experiments performed in vivo on the kinetics of the induction of β-galactosidase (Pardee and Prestidge, 1961; Kepes, 1962) (see figure 6) or on histidase (Hartwell and Magasanik, 1963) as well as on the effects of actinomycin in the induced synthesis of proteins (Levinthal et al., 1963) show that the induction of an enzyme involves first, before any synthesis of the specific protein occurs, the accumulation of a constituent which, it seems, can only be the messenger.

By these two series of observations we can conclude that the phenomena of repressive regulation certainly govern the concentration of different types of messengers in the bacteria. A priori, this effect can be the result of a synthesis or of a differential stabilization of messengers. Although a mechanism probably exists in bacteria which permits the destruction of defective messengers or those which are not read (cf. Attardi et al., 1963; Beckwith, 1964 a), there are a great number of arguments against the hypothesis of an accelerated destruction of messengers under the effect of repression. But if the mechanism of repression appears to govern the coding process, that is to say the biosynthesis of the messenger, we must still identify the operator, that is to say the place of action of the repressor. It is possible, in fact, and many observations lead in this direction, that the coding and translation processes are not independent. One can then theorize that a repressor acting on the level of translation, for example on the level of the messenger polysome complex, could inhibit the synthesis of the corresponding messenger by a retroactive effect.

Before pursuing this discussion, it will be necessary to summarize here the recent results which permit us to state precisely the localization and the function of the operator locus.

1. Deletions have been isolated in the structural gene of β-galactosidase covering the known mutations situated

nearest the operator locus. These deletions do not affect the inducibility of two other proteins of the operon, permease and transacetylase (Beckwith, 1964 b). Inversely, the deletions covering the operator locus but respecting the gene for β-galactosidase, which involves a synthesis forming the enzymes of the operon, do not modify the maximum rate of enzyme synthesis nor, it seems, the structure of the β-galactosidase formed according to the criteria of temperature sensitivity, pH etc. (Jacob et al., 1964). These two observations show that, contrary to what was possible to suppose before, the operator segment is distinct from the structural cistron which is immediately adjacent to it and does not participate in the determination of the structure of the corresponding protein or of any other protein of the operon.

2. Isolation of a series of constituent mutants (o^c) in various conditions has shown that these mutations are produced simultaneously or under the influence of X-rays, but not by the action of 2-aminopurine. The o^c mutations which are always localized in the lactose segment of the chromosome and cannot, therefore, be attributed to an external suppressor, do not seem to revert to the wild type ($o^c \rightarrow o^+$). Finally a single mutation often affects (15 to 20% of the cases) not only the operator, but also the neighboring regulatory gene i (see figure 5). All of these observations seem to indicate that the o^c mutations result exclusively in deletions originating more or less from a segment situated outside the structural genes.

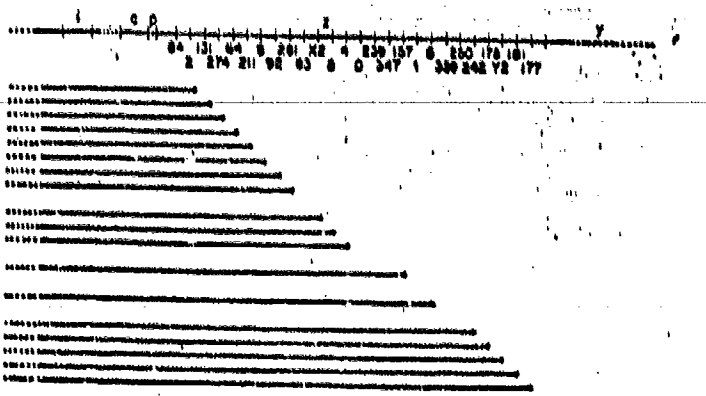

Fig. 8. Deletions affecting the z gene and the operator of the lactose region at the same time

Mutations (i^S) are known which involve the formation of a modified repressor which the β-galactosidases can no longer inactivate. The i^S mutants are incapable of forming the proteins of the lactose operon. The capacity to form these proteins can be recovered by the secondary mutations which, in the diploids $i^S/F\ i^S$, are structural operator mutations o^c. In growing these diploids $i^S/F\ i^S$ on melibiose one can select secondary mutants o^c which can form β-galactosidase-permease (likewise utilized for the transport of melibiose). Among the isolated secondary mutants 97% are Lac^+ and 3% are Lac^-. These last ones are all carriers of deletions which extend to the left farther than i in every case, and to the right more or less far toward the z gene determining β-galactosidase. The first line on the top represents the chromosome and each number corresponds to an isolated deletion coming from the mutant i^S, the extension of which has been measured by a series of crosses with point mutations. Although a segment of z gene situated next to the operator may have disappeared, many of these deletions produce fragments of proteins capable of "complementing" the point mutations of the β-galactosidase and of thus producing an active enzyme. The z gene then contains several cistrons, at least 2 and probably more.

3. In selecting secondary mutants capable of forming galactoside-permease from certain mutants which cannot be induced by β-galactosides, it is possible to isolate deletions covering o and a fraction of the structural gene adjacent to z at the same time, thus determining β-galactosidase. These mutants show very definite properties: the deletion can, if adjacent to z, be stopped in any region of the cistrons forming the z gene; but in every case analyzed (more than 80) it extends, from the other side, farther away than the regulatory gene i (see figure 8). Moreover, the production of other proteins of the operon (permease and transacetylase), which is determined by the genes left intact by the deletion, represents no longer, in the presence as well as in the absence of β-galactoside inducers, 10 to 20% of the one observed in the induced wild type (Jacob et al., 1964). These observations show that between the operator segment and the beginning of the so-called operon, (that

is to say the proximal end of the first structural cistron) a particular segment intervenes for which we have proposed the term "promoter" (p).

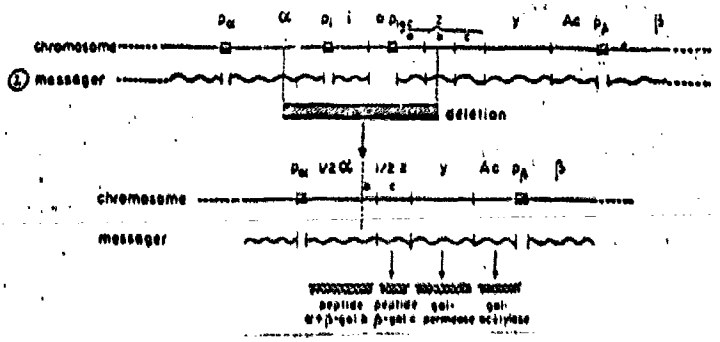

Fig. 9. Scheme of the deletions affecting the proximal fragment of the lactose region

[Legend]: 1) messenger.

On the chromosome the lactose region is represented with the regulator gene i, its promotor, operator o, promotor p of the lactose operon and the structural genes z (with three cistrons), y and Ac. To the left and to the right two other unknown operons are represented, which are designated as α and β, with their promoters p_α and p_β. The lactose operon is coded into an integral messenger forming the three proteins. The regulator gene i is coded into a messenger which will form the repressor. The two operons α and β are each coded into a messenger. The deletion represented corresponds to one of the deletions described in figure 8 and in the text. It affects the terminal fragment of operon α, the gene i, the operator and promotor of the lactose region, as well as all of the first cistron a and the segment of the second cistron b of the z gene determining β-galactosidase. The initial segment remaining of operon α becomes fused by the deletion with the terminal segment which remains of cistron b. A single messenger is then formed comprising half of α and half of b and all the other intact structural elements of the lactose operon (the cistron

c of z and the genes z and Ac). A peptide is probably formed corresponding to the beginning of α and to the end of b. The peptides c, y and Ac are normally formed, but at a rate which is now governed by the promoter (and possibly the operator) of α and which is independent of the action of the β-galactosides, since the inductor effect of these is normally exerted on the regulation cycles determined by the regulator gene i and the operator of the lactose operon, both of them having been destroyed by the deletion. All the deletions of this type extend farther than i, probably because i functions too slowly in order to produce permease in sufficient quantity after a fusion of i and z.

The deletion of the promoter brings about the inactivation of the entire operon, and as a result of a chromosomic change, this operon does not become fused with another operon depending upon another promoter (see figure 9).

These observations, and notibly the fact that these o^c mutations are neither induced by 2-aminopurine nor are they products of external suppressors, show that the operator segment is not translated. These facts do not let us affirm, however, that the operator segment is no longer coded. One must note here that the manner in which the messenger is synthesized is still unknown, that is to say, whether the coding of the operon begins on the side of the operator, like the translation of the messenger in the peptide chain, or on the other side. If the code begins on the side of the operator, the properties of the different known deletions indicate that, according to all probability, the code must begin at the level of the promoter. The operator would not be coded and the repression would then act directly on the DNA in order to block the code. If, on the contrary, the code begins at the opposite extremity, the repressor would block the liberation of the messenger and thus its production, by acting on the DNA if the operator is not coded, or on the RNA if it is coded. In the latter case the promoter could correspond to a segment or would necessarily begin the translation of the messenger by the ribosomes. In the absence of chemical knowledge which would permit us to establish a correlation between extremities of the messenger (for example 5'P) and those of the peptide chains (for example NH_2 of the proximal chain), it is necessary to stress that in systems known at the present time, the deletions affecting one or

the other of the extremities of the operon have very different effects: contrary to deletions affecting the promoter, the deletions affecting the other extremity do not modify the expression of the cistrons of the operon still intact and, in particular, do not change the maximum rate of production of the corresponding proteins.

The hypothesis which lets us consider immediately, and at the same time in a simple manner, the experiments involving the kinetics of induction and the properties of various mutants, seems to be that the operator locus constitutes by itself an element at the level from which the represser acts directly by associating with it, and would block the processes of transcription beginning at the level of the promoter segment. Whatever it is, it appears certain that the repressor governs the integral coding of an operon directly on the genetic level or indirectly on the polysome level.

E. Punctuation of the Deoxyribonucleic Text

The properties of the operon as a unit of genetic expression coded into an integral messenger, containing information for many peptide chains, poses an important problem that we shall discuss here, namely that of "punctuation" in the code and the translation of the linear chemical text represented by DNA.

The DNA contained in the bacterial chromosome is made up of very long molecules in a double chain (probably only one) corresponding to a large number of operons. It seems that each operon may be coded independently and that the RNA polymerase codes only one of the two sequences of DNA (Marmur et al., 1963; Spiegelman and Hayashi, 1963; McCarthy and Bolton, 1964). We must therefore admit that certain segments of the sequence determine, on the one hand, which one of the DNA chains should be coded and define, on the other hand, the beginning and the end of an operon, representing the points where the code in the messenger RNA of the chosen DNA chain begins and ends. Since the messenger RNA corresponding to the operon can perhaps be translated itself into several distinct peptide chains, it is likewise necessary that this messenger (and consequently the DNA of which it is the transcription) expresses elements of interpretible sequences by the system of translation as interruptions between peptide chains.

It is necessary, therefore, to admit that DNA expresses at least 2 punctuation systems, of which one is interpreted at the level of code by the RNA-polymerase, while the

second is expressed at the time the messenger is translated into peptide sequences. Before describing of what these punctuation signs could consist chemically, we would like to propose an exact set of symbols which will define the problems and the hypotheses.

For the punctuation of the code we shall adopt the opening and closing signs of parenthesis to symbolize the beginning and end of the integral messenger. We shall see immediately if these two signs are necessary and if they are sufficient.

For the punctuation of the translation, we shall adopt commas as signs marking the interruptions between successive and distinct peptide sequences. We shall see whether certain facts or hypotheses make the introduction of other punctuation marks besides the comma necessary. We shall use the colon for symbolizing the beginning of the translation of the integral messenger, and the semicolon in order to differentiate certain kinds of interruption between peptide sequences.

Let us consider first the punctuation of the code the preceding discussion concerning the structure of the lactose operon and, in particular the relationship between the structural genes of this operon and the operator, lead to two essential conclusions, namely:

1. The operator corresponds to a non-translated segment;

2. Between the operator and the beginning of the structural cistrons a particular segment, the promoter, governs the expression of the entire operon.

We have likewise seen that these results are open to two interpretations (see figure 10). According to the first hypothesis, the segment corresponding to the operator would be coded, but it would not be read. It has hypothesis is accepted, a single punctuation mark, for which we will adopt the opening sign of a parenthesis, would be necessary in coding (figure 10 b). This sign would mark at the same time the beginning of the code of an operon and the end of the code of the preceding operon. The promoter, in this case, would represent a punctuation of the translation for which we shall adopt the sign of the colon.

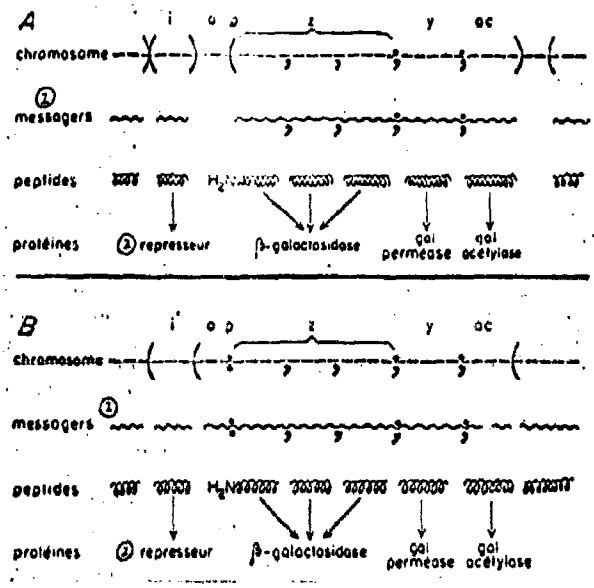

Fig. 10. Hypothesis on the punctuation of the nucleic acid text

[Legend]: 1) messenger; 2) repressor.

The scheme represents the lactose operon of E. coli, the regulator gene i, and other operons, unknown on both sides. The opening and closing signs of the parentheses correspond to the punctuation on the DNA code in the messenger, while the comma, semicolons, and colons represent the punctuation of the translation in the peptide chain. In hypothesis A, operator o is not coded and the regulation acts directly on DNA. The code beings at the level of promoter p which corresponds to the opening sign of the parenthesis. The code of the messenger follows the length of the structural genes (z: β-galactosidase comprising 3 cistrons, y: permease and Ac: transacetylase) up to the closing sign of the parenthesis. In hypothesis B the transcription begins at the level of the opening sign of the parenthesis. The operon is coded and the code continues to the opening sign of the next parenthesis. In this scheme the repression acts on the operator segment of the messenger which is not translated. Besides

the commas marking interruptions in the peptide chain, another punctuation mark is necessary, i.e., the colon, which marks the region where translation by the ribosomes begins. In these two cases a comma indicates an interruption of the peptide chain without an abatement of translation while a semi-colon represents an interuption accompanied by an abatement of translation as is the case with transacetylase. It is possible that a single semi-colon and not two, are sufficient between z and Ac.

In the second hypothesis, which, we believe, is the more probable, the operator is not coded. The beginning of the code would then correspond to the promoter which would, therefore, represent a punctuation of the code which is symbolized again by the opening sign of a parenthesis. But it is clear that this hypothesis requires the use of another punctuation mark marking the end of the code of the operon (figure 10 a). In this system the DNA segments included between the parentheses correspond to the operons (defined as segments coded in an integral manner), while the segments outside the parentheses correspond to operators.

Let us consider now the punctuation of the translation. We have already stressed the fact that in the hypothesis where the operator segment is coded, the promoter must be interrupted as a punctuation of the translation marking the beginning of peptide synthesis. In the second hypothesis, on the contrary, no particular punctuation sign is necessary to mark the beginning of the translation which would commence at the very end of the polynucleotide messenger. In all the hypotheses, a sign of interruption between the segments corresponding to different peptides is indispensible; we shall employ the comma (see figure 10) for this sign.

It is necessary however to draw attention to the fact that the punctuation signs effectively employed in DNA and RNA messengers could function not only in dividing up the code and translation, but also in determining, everything else being equal, the speed or at least the maximum speed of the corresponding processes. This applies in particular to the opening signs of the parenthesis marking the beginning of the code. Certain operons are codable at a maximum speed, this speed being governed in another way by the process of repression. This is the case for example in the lactose operon. But other operons can be imagined which could

function at a slow, constant speed which is independent of all regulation. This could be the case with segments governing the synthesis of repressors themselves, that is to say, regulatory genes. The chemical punctuation corresponding to the opening sign of a parenthesis could then be differentiated quantitatively, according to the maximum coding speed which this sign permits.

Remarks on the same order must be made concerning the signs of interruption of peptide sequences interpreted in translations. One knows in fact that in the same operon the enzymes or peptides corresponding to distal segments in regard to the operator are synthesized in a smaller quantity than the peptides corresponding to proximal segments (Zabin, 1963; Ames and Hartman, 1963; Burstein et al., 1964). It is therefore a case of a quantiative polarization of the expression of the operon, a polarization marked more or less according to the circumstances which could correspond to different punctuation marks. On figure 10 an interruption of the peptide chain, accompanied by a decrease in the rate of synthesis of the dital peptide chains, is represented by a semicolon.

One can see that the different punctuation marks which we have defined by the proposed symbols constitute merely the indispensable minimum in the present state of our knowledge on the coding and translation processes. It seems probable enough that later the number of these marks will have to increase. We still have to find the structures or whatever corresponds chemically to these various punctuation signs.

The opening sign of a parenthesis marking the beginning of the code must, in all likelihood, be interpreted by the RNA polymerase. One of the hypotheses which is presented along these lines is that this sign could consist of an interruption of a covalent bond carried on one of the two DNA strands. This would at the same time explain the choice the DNA strans to be coded and the segmentation of the code in the operons. It is likewise possible that this sign might be composed of a particular nucleotide sequence (perhaps a repitition) and one could easily theorize that very specific and extensive methylation of DNA (Gold et al., 1963) is utilized for this punctuation. Let us note that in all cases, if the punctuation effectively recognized by RNA-polmerase in an interruption, this break of the chain could have been created only after the replication of DNA and must, in this case, be due to the recognition of a particular punctuation

by one or several specific endonucleases. Similar hypotheses can be made in which the sign of the end of the code is concerned.

Since the reading of the messenger is integral and has been affected by the intermediary of transfer RNA, it is extremely tempting to suppose that the signs of an intteruption between peptides are recognized by particular RNA's which could, for example, not transfer specific amino acids but be specific for the transfer of N-acetylated amino acids. One can hope to obtain quite rapidly a confirmation of this hypothesis. If, in fact, the commas were read by the particular transfer RNA's, a change of phase to the reading resulting from the deletion or the insertion of one or several nucleotides (in numbers which are not multiples of three), would, as has been shown by Crick and his collaborators (1963), involve a disturbance and miscoding not only in the peptide governed by the corresponding segment, but in all the following peptides in the reading of the messenger. In other words there where they match, the commas would probably be abolished, but the signs of interruption could appear in some other points. The predictions founded on this hypothesis are, therefore, precise enough so that they could be subjected to further investigations.

To conclude, let us say that our intention in this discussion was not to propose a solution to the problem of the punctuation in genetic information, but to show that this problem has been defined in an exact manner as a result of recent progress.

IV. Regulation of Synthesis of DNA and of Cell Division

Through the properties of allosteric protein we understand how the metabolic pathways in the cell are regulated and coordinated at every moment. By the mechanism of genetic repression we have learned to understand the regulation of the biosynthesis of proteins. The essential biological phenomenon, for which the whole chemistry of the bacterial cell prepares itself, is the replication of DNA which, in turn, permits the reproduction of the cell. Evidently a close connection exists between DNA replication and so-called cell division. Before discussing the mechanism of this association, we shall describe the regulation of DNA replication.

A. The Replicon, a Unit in DNA Replication

Genetically, the hereditary material of a bacterium is composed of an integral structure, the bacterial chromosome, which is the unit of segregation (Jacob and Wollman, 1961). Chemically the DNA of this chromosome certainly seems to be an integral structure itself. In fact, it appears (see Thomas, 1963), that the DNA of a bacterial chromosome, as well as viral DNA, is not formed by a mere assemblage of molecules of associated polynucleotides together with other constituents, but by only one double nucleotide sequence, which is unified in such a way as to form a ring. This ring structure, which was first proposed on the basis of genetic results (see Jacob and Wollman, 1961), has been proved directly by the elegant experiments of Cairns (1963).

The very existence of this chemically integral structure suggests that its replication must be an integral process. This appears to be confirmed by various experiments involving biochemical methods (Maaloe, 1961; Lark, 1963), physico-chemical methods (Meselson and Stahl, 1958; Bonhoeffer and Gierer, 1963), genetic methods (Sueoka and Yoshikawa, 1963) or even by direct observation (Cairns, 1963). The replication of DNA seems to begin at a given point of the structure and progresses regularly along the length of this structure during the majority of the generation time; no cycle of replication can begin before the preceding cycle has ended.

The unitary replication of such an integral structure must obey a unitary regulation. This problem can be attacked in bacteria thanks to the existence of episomes, the genetic elements which can multiply in the bacteria in an independent fashion or as a fragment of bacterial chromosomes after becoming attached to it (Jacob and Wollman, 1961). This is the case for example of λ phage. After infection, the DNA of this phage determines the synthesis of protein precursors and then multiplies very rapidly independently of the cellular system regulating the replication of chromosomal DNA; this vegetative multiplication results in the production of infectious particles. In lysogenic bacteria, on the contrary, the phage DNA is inserted as prophage in the bacterial chromosome. It then submits to the regulation of this chromosome and is only replicated once by the bacterial generation. The episome F, which in E. coli determines the sexual type, behaves in a similar fashion. It is autonomous in the males called F^+, and multiplies independently of the bacterial chromosome, in certain conditions even faster than the bacterial chromosome. Among the males called Hfr, on the

contrary, it behaves like prophage, and is attached to the bacterial chromosome and submits to its regulation. The DNA of these two episomes have been isolated and purified. This DNA also clearly seems to be composed of an integral molecular structures, probably in the shape of rings (cf. Campbell, 1962).

And so the same unit of DNA is capable of replicating itself at different speeds depending on the system of regulation by which it is governed. Contrary to what is observed in vitro, (cf. Kornberg, 1961), the capacity of replicating itself in vivo is not a matter of any deoxynucleotide sequence, but involves the integral structures which are the chromosomes and the episomes. This point is illustrated by the behavior of sequences such as the ones composing the lactose or galactose segments in E. coli. These segments have normal elements of the bacterial chromosome and replicate themselves therefore once in a generation. As a result of a kind of genetic recombination they can be incorporated into the DNA of an episome, the lactose or galactose segments are replicated whenever the episome replicates itself independently of the bacterial chromosome, i.e., when the episome is autonomous. These same lactose or galactose segments are, however, incapable of replicating themselves in isolation when they are not part of an integral structure, like the chromosome or the episome; a fragment of DNA from the bacterial chromosome of a donor bacterium transferred by conjugation or transformation into a receptor bacterium is then able to recombine with a chromosome, but is not able to replicate itself as such.

All of these observations lead to the conclusion that the integral molecular structure, which the DNA of chromosomes or of bacterial episomes represents, constitute units of replication for which we have proposed the term replicons, the unitary properties of which depend on the presence and activity of certain specific determinants carried by the replicon (Jacob et al., 1963). In other words, each replicon determines a specific system which governs its replication as illustrated by the behavior of phage. In the prophage state, the DNA of the phage is replicated with the bacterial chromosome once by division, and the expression of the phage genes is inhibited by a specific system of repression. In order that this DNA can multiply itself in an autonomous fashion it is necessary that the repression be raised so that, before any replication takes place, synthesis of precursor proteins can occur; an example of one such precursor protein is a nuclease which has been discovered very recently (Weissbach and Korn, 1962).

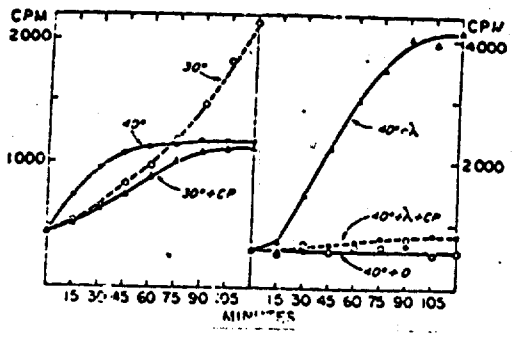

Fig. 11. Incorporation of radioactive thymine at high and low temperature into the CRT83 mutant under different conditions

The CRT83 mutant, isolated from existing bacteria in thymine, forms colonies at 30°C, but not at 40°C. The bacteria are cultivated at 30° in a minimal medium plus casamino acid and non-radioactive thymine. At zero time for each experiment, the bacteria are centrifuged, washed, and re-suspended in the same environment containing thymine C^{14}. At different times trichloroacetic acid). The millipores are then dried and counted.

On the left: The bacterial suspension was divided into three fractions, one agitated at 30°C, the second at 40°C, and the third at 30°C, in the presence of 30 γ of chloramphenicol. One can see that at 30°C the bacteria synthesize RNA exponentially; at 40°C they synthesize it only for about 45 minutes and the quantity syntehsized is the same as at 30°C in the presence of chloramphenicol.

On the right: The bacteria previously maintained one hour at 40°C were infected with the λ phage (at zero time) They were agitated at 40°C with or without chloramphenicol. When the non-infected controls do not synthesize DNA, the infected bacteria do synthesize it and the production of phage is nearly normal. In the presence of chloramphenicol the infected bacteria do not synthesize DNA in a significant quantity (according to Kohiyama et al., 1963)

One of the immediate predictions of this hypothesis is that in each DNA unit certain mutations should affect the system of replication and, consequently, inhibit specifically the replication of the mutated unit. Such mutations would be lethal since they would affect the essential functions of causative genetic elements and possibly cell division. One would only be able to isolate mutants of this type if their effects were manifested only under certain conditions, for example, at a high, but not at a low temperature. It has been thus possible to isolate the mutants of E. coli in which the replication of one of the known genetic units is specifically inhibited.

1. Among a series of bacteria mutants unable to multiply at a higher temperature, there is a type in which the replication of the chromosome is inhibited by a high temperature. The bacteria multiply normally at 30°C. Placed in an environment of 40°C they are able to complete the cycle of chromosomal replication which had already started but are not able to begin another one (see figure 11). In these bacteria the mutation seems to affect the production of a protein, by which one can show that it is not the DNA polymerase, but something which appears necessary to the replication of only the chromosome. In fact, the bacteria left at a high temperature for a sufficiently long time so they will no longer synthesize bacterial DNA can still synthesize the DNA of the λ or T6 phage which they normally reproduce (Kohiyama et al., 1962). It seems then, although the production of a protein may be necessary for the beginning of the replication of the bacterial chromosome, as Maaloe (1961) had already suggested, that this protein may be distinct from the DNA polymerase.

2. In the phage certain mutants affect the precursor functions of the phage. The DNA of the mutated phage can still replicate itself as integrated prophage, but not in an autonomous the alteration seems to affect the formation of a diffusible substance since the presence in the same bacteria of another phage genetically intact permits the assurance of the replication of two mutated and intact DNA's. In certain of these mutants, the diffusible product had been identified with the proper nuclease of the phage (Redding, 1964).

3. Certain mutations of the sexual episome specifically prevent the autonomous reproduction of this episome at high, but not at low temperatures, without affecting the multiplication of the host bacterium. The autonomous replication alone of the sexual episome appears inhibited at a high

temperature, for after attachment to the chromosome in the Hfr state, the episome is multiplied normally at all temperatures. Even there, it still seems reasonable that the mutation affects a diffusable product because the autonomous reproduction of the mutated episome is made possible at high temperature by the presence of a second sexual, genetically intact episome in the same bacteria (Jacob et al., 1963).

These observations show that, according to the predictions drawn from the hypothesis of the replicon, each DNA unit possesses determinants whose expression governs the autonomous replication of this unit. Two of these units can fuse into a single one whose replication is then governed by the determinants of only one of the fused units. In order to take account of these observations, it is necessary to admit that a system governing the replication of a DNA unit contains, like the cycles insuring the regulation of protein synthesis, at least two elements: one specific diffusible element, probably a protein, the structure of which is determined by a segment of a DNA unit, and an element of recognition which is nondiffusable and inscribed into the DNA sequence. The properties of the replication mutants suggest that the diffusable element plays the role of activator in the replication (in opposition to the role of inhibitor which the repressor plays in the coding). The term initiator has been proposed to designate the diffusable activator element and that of the replicator for the designation of the recognizable element corresonding to the punctuation of the replication (Jacob et al., 1963). It is still not possible to assign a precise chemical role to the initiator. It could be either a specific DNA polymerase, or rather an enzyme capable of specifically recognizing the holomogous replicator and, for example, of opening at this level the ring structure, thus permitting the DNA-polymerase to act and copy the entire deoxynucleotide sequence inserted after the replicator.

It is at the level of such regulatory cycles which specifically govern the replication of an integral structure of DNA, that the interaction of signals takes place which permit or prohibit the beginning of the replication of this structure in order to coordinate the replication with the cell division. This is the problem which we shall now discuss.

B. Replication of DNA and Cell Division

It has often been postulated that the metabolites intervening in the DNA synthesis must play a regulatory role in this synthesis (cf. Lark, 1963). A metabolic regulation must surely occur, but this does not seem sufficiently specific and exact to take into account the co-ordination observed between DNA replication and cell division. Such a coordination involves the existence of close connections uniting the two processes. In other words, in bacteria which seem to be deprived of the complicated mitotic apparatus which is observed in cells of higher organisms, a mechanism is necessary which insures the regular segregation of the long DNA strands into the daughter cells which are formed at each division.

By taking into account the properties of the sexual episome of E. coli, the study of this problem can be approached. The transfer of genetic material from the male to the female is determined and guided by this sexual episome which is present in each bacterium is always very small (on the order of one per chromosome), the transfer of genetic material is very efficient. It takes place in nearly 100% of the conjugating pairs, even when in the same conditions the cytoplasmic constituents are not transferred. It is necessary, therefore, to conclude that the sexual episome occupies a privileged position very near to the cell membrane in the bacterium, and that it is, according to all probability, attached to it. This conclusion leads quite naturally to the hypothesis according to which all the genetic nonviral elements of the bacterial cell, chromosomes or episomes, are attached to the cell membrane. One such hypothesis would permit us to shed light on the coordination between DNA replication and cell division, and the regular separation of DNA in bacteria (Jacob et al., 1963).

The existence of a close relationship between the cellular membrane and the bacterial chromosome has been demonstrated by direct observation of B. subtilis under the electron microscope (Ryter and Jacob, 1963). This organism contains a small number of structures called mesosomes which are formed by the investigation of the cell membrane and in which is found, as in the membrane itself, the energy generator system. Each chromosome appears attached to one of these mesosomes and the tie is sufficiently strong so that in plasmolysis the mesosome, which is progressively evaginating, draws with it the chromosome which then appears closely attached to the membrane (see figure 12). In other words, it

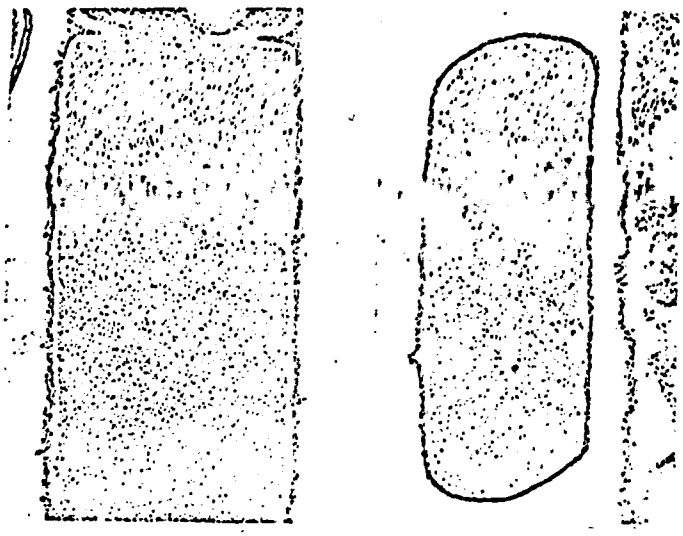

Fig. 12. Section of B. subtilis examined under an electron microscope

To the left: growing bacteria. The nucleus is joined to a mesosome, the lamellar structure itself being attached to the cytoplasmic membrane.

To the right: plasmolysed bacteria after having been placed for 30 minutes in a medium containing 2M sucrose. The mesosomes are expelled and form small vesicles near the membrane. While retracting, the mesosome, being joined to the nucleus, draws the nucleus with itself, and the nucleus thus appears directly united to the membrane by a very fine bridge (according to Ryter and Jacob, 1963).

seems very reasonable that the two chromosomes formed by replication are separated from one another by the division of the initial mesosome. Each one of the chromosomes formed remains attached, in fact, to one of the mesosomes. These appear to separate gradually, each one carrying along its chromosome; this separation process probably occurs as a result of the formation of a cell membrane which progressively forms between the two mesosomes (see figure 13). Since a division septum is ultimately formed in the region of the membrane which then separates the two mesosomes, it is clear that this mechanism insures separation and a regular distribution of two chromosomes between the two bacteria thus formed.

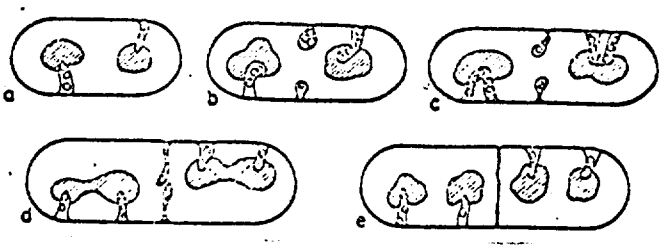

Fig. 13. Diagram of the division process in B. subtilis

This diagram is drawn from the observation of sections of B. subtilis under the electron microscope. The growing bacteria have been fixed and imbedded in the vestopal. Series of successive sections 1,000 A in thickness were obtained. This thickness permits a resolution sufficient to sharply distinguish the mesosomes and the nuclei. When the section is nearly parallel to the longitudinal axis of the bacterial cell, about 6 or 7 sections are obtained of the same bacterium. The contours of the mesosomes and the nuclei observed in each section are laid out on a transparent paper and by superimposing the sections of one bacterium, it is possible to make a three dimensional model of this bacterium. Twenty different bacteria have thus been examined. The scheme represents the various stages of the division process as it appears in these bacteria. The shaded regions represent the nuclei. The ones containing the circles represent the mesosomes.

At the beginning of the cycle each nucleus is connected to a ribosome. The nuclei are then magnified while the mesosomes appear which insure the formation of septum. The nuclear mesosomes are split into two and turned away one from the other, each forming a new nucleus as it pursues the formation of septum. When this is achieved, each of the two bacterial cells contains two small nuclei, each linked to a mesosome (Ryter and Jacob, 1963).

The same reasoning can be applied to each of the bacterial replicons (nonviral chromosomes or episomes). According to

this hypothesis it is an element of the membrane, perhaps the same mesosome to which would be attached the cellular replicons, which represent in the bacteria the units of segregation and which are comparable to a chromosome in higher organisms.

In order that DNA replication and cell growth may be coordinated in this diagram, it appears necessary that the signals permitting or prohibiting the replication, act on the level of the zone of attachement. This is what the phenomena of bacterial conjugation also suggests. The contact between certain antigens of the male bacterial surface and that of the female starts into motion the formation of a protoplasmic bridge by which DNA segments can be transferred from the male to the female. Although the exact nature of the mechanism insuring the transfer of DNA is still unknown, it appears clear that a signal coming from the bacterial surface sets off a series of reactions which definitely cause the nuclear phenomenon of mobilizing certain DNA elements. The initiation of this transfer, determined and guided by the sexual episome, is sensitive to small concentrations of acridine, which is known to differentially inhibit the autonomous replication of the sexual episome (Hirota, 1959; Jacob et al., 1963). These effects of acridine suggest that the signals which in the sexual episome start either the replication or the transfer by conjugation, have a common origin and permit the interaction of the common elements probably occuring in the cellular membrane.

This conclusion, if it can be generalized to different bacterial replicons and notably to the chromosomes, should help us understand the mechanism which insures coordination between growth and DNA replication in bacteria. It is at the level of the cellular membrane where the signal is given which starts the replication cycle of DNA by intermediate regulatory pathways. While this replication cycle is in operation, the synthesis of the membrane between the points where the nucleic structures thus formed are attached, would insure their progressive separation. A new replication cycle would be prevented until the growth of the membrane, which is itself coordinated with other syntheses, has reached a certain point. The membrane would then transmit the signal permitting the beginning of a new replication cycle. It is at the level of the membrane, which is newly synthesized between the attachment zones of DNA, where the new division septum is formed. The formation of the septum and the separation of the bacteria cannot constitute the stages of the sequence of reactions postulated for the lengthening of the

membrane and the replication of DNA, but they must be coupled with these processes because certain treatments (UV light for example), or certain mutations, inhibit the formation of the septum and the division without blocking for long the lengthening of bacteria nor the synthesis of DNA.

V. Conclusions

One can try to sum up in a synthetic fashion these conclusions to which the study of the mechanisms of regulation in bacterial cells leads.

DNA performs two essential functions: replication and coding, the latter being followed by the translation of the chemical text. These two functions permit the interaction of acting elements, but in each of them DNA intervenes directly as a matrix and indirectly as an emitter and receptor of specific chemical signals by which these two functions are regulated.

As an emitter, DNA acts on all levels, it seems, by defining the primary structure, and therefore the properties of the proteins carrying out specific regulatory functions. It is through the action of these proteins that all the regulatory actions necessary for proper cellular functioning are established and relayed.

As a receptor, the role of DNA appears to be strictly passive. The specific sequences defining the operators and the punctuations of the code or the replication seem to be recognized, whether masked or uncovered, by the regulatory proteins themselves.

According to this scheme all the systems of cellular regulation could be definitively represented by a cycle tying the responsible segment for the structure of a regulatory protein to another segment of DNA which is recognized by the protein and subject to this regulation. It is through the action of the intermediary of such a cycle that chemical signals coming from the cytoplasm of another cell or from the medium can act on the DNA.

If each of the regulatory routes were independent of the others, coordination of different regulatory processes would not be insured. One can also see that each regulatory cycle is, in fact, associated with one or several others through the intermediary of the metabolites produced by a

system, but recognized as regulatory signals (that is to say as allosteric effectors) by one, or possibly several different systems.

One can, therefore, state with some degree of exactness, that DNA constitutes a structural "memory." But since the cellular structures are comprised of those molecular structures which are directly responsible for the regulatory interaction, one can likewise state with the same degree of exactness that the structural memory of DNA comprises a program of regulation permitting coordinated utilization of this memory.

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